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Vicinal Dithiol–Disulfide Distribution in the *Escherichia coli* Mannitol Specific Carrier Enzyme II^{mtl} †

F. F. Roossien and G. T. Robillard*

ABSTRACT: *Escherichia coli* mannitol specific EII in membrane vesicles can be inhibited by the action of the oxidizable substrate-reduced phenazine methosulfate (PMS) in a manner similar to *E. coli* enzyme II^{Glc} [Robillard, G. T., & Konings, W. (1981) *Biochemistry 20*, 5025–5032]. The fact that reduced PMS and various oxidizing agents protect the enzyme from inactivation by the sulfhydryl reagents *N*-ethylmaleimide and bromopyruvate suggests that the active form possesses a dithiol which can be protected by conversion to a disulfide. The sulfhydryl–disulfide distribution has been examined in purified EII^{mtl} by labeling studies with *N*-[1-^{14}C]ethylmaleimide ([^{14}C]NEM). EII^{mtl} can be alkylated at three positions per peptide chain. When alkylation takes place in 8 M urea, only two positions are labeled. The third position becomes labeled in urea only after treatment with DTT, suggesting that the native enzyme is composed of two subunits linked by a disulfide bridge. The remaining two sulfhydryl groups per peptide chain appear to undergo changes in oxidation state as indicated by the following results. (1) Treatment of the active enzyme with NEM leads to complete inactivation and incorporation of 1 mol of [^{14}C]NEM per peptide chain. Oxidizing agents protect the activity and prevent labeling presumably by forming a disulfide. (2) Phosphorylating the enzyme (one phosphoryl group per peptide chain) fully protects the activity, but 1 mol of NEM per peptide chain is still incorporated. Subsequent dephosphorylation by adding mannitol causes a second mole of [^{14}C]NEM to be incorporated and results in complete inactivation. (3) The site that is labeled in the phosphorylated enzyme can also be protected by oxidizing agents. The possibility that dithiol–disulfide interchange occurs during the turnover of the carrier is discussed.

The activity of the glucose-specific carrier from *Escherichia coli* $EIIB^{Glc}$ [1] is controlled by the redox potential. At low potentials $EIIB^{Glc}$ is in an active high-affinity form in which two vicinal thiol groups are reduced. At potentials greater than approximately −100 mV the dithiols convert to a disulfide (Robillard & Konings, 1981). Similar observations have been made for the lactose and proline carriers in *E. coli* which are totally different from the glucose carrier in their mode of energization (Konings & Robillard, 1982). On the basis of these data and similar observations for other transport and energy transducing systems, we suggested that dithiol–disulfide interchange could play a general role in energy-dependent processes (Robillard & Konings, 1982). Whether the redox changes serve a regulatory function or whether they are actually involved in the turnover of the enzymes has remained uncertain. The study presented in this report attempts to address this question for one transport system by examining the distribution of dithiols and disulfides in a purified EII and the various intermediates which occur during a turnover. The mannitol-specific EII has been used.

Recently a purification procedure for EII^{mtl} has been published (Jacobson et al., 1979). It is not known whether subunit interactions are involved in the catalytic activity of the enzyme. Kinetic studies have revealed that both EII^{mtl} and EII^{Glc} catalyze the phosphorylation of their respective substrates by ping-pong mechanisms involving P–EII intermediates as indicated in Scheme I (F. F. Roossien, M. Blaauw, and G. T. Robillard, unpublished results; Misset et al., 1983; Rephaeli & Saier, 1980). The distribution of redox states has been determined in EII^{mtl} and $P–EII^{mtl}$ before and after reaction with mannitol by monitoring the extent of incorporation of [^{14}C]NEM in these intermediates. The data obtained indicate that the active enzyme possesses two sets of vicinal thiols whose reactivity toward NEM alternates with the phosphorylation state of the enzyme. One set is reactive in the dephosphorylated enzyme but not in the phosphorylated species. The other set has the opposite reactivity. Both sets can be protected by oxidizing agents or dithiol specific reagents.

Scheme I

$$P–HPr + EII^{mtl} \rightarrow P–EII^{mtl} \xrightarrow[mtl]{} EII^{mtl} + mtl\text{-}1\text{-}P$$

$$P–EIII^{Glc} + EIIB^{Glc} \rightarrow P–EIIB^{Glc} \xrightarrow[Glc]{} EIIB^{Glc} + Glc\text{-}6\text{-}P$$

Experimental Procedures

Materials

[1-^{14}C]PEP (monocyclohexylammonium salt; specific activity 12 mCi/mmol) and D-[1-^{14}C]mannitol (59 mCi/mmol) were obtained from Amersham. *N*-[1-^{14}C]Ethylmaleimide (23.7 mCi/mmol) was from New England Nuclear. Lubrol PX, sodium deoxycholate, hexylagarose, and butylagarose were from Sigma. Sodium deoxycholate was recrystallized twice from acetone/H_2O. All other chemicals were reagent grade from commercial sources.

EI and HPr were purified from *E. coli* P650 as described previously (Dooyewaard et al., 1979; Robillard et al., 1979).

Methods

Growth Conditions. Escherichia coli ML 308/225 was grown under aerobic conditions at 37 °C in medium 63 (Saier

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[1] Abbreviations: PMS, phenazine methosulfate; PEP, phosphoenolpyruvate; $EIIB^{Glc}$, glucose-specific enzyme II; EII^{mtl}, mannitol-specific enzyme II; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; TDL, buffer containing 20 mM Tris-HCl, pH 8.4, 1 mM DTT, and 0.05% or 0.5% Lubrol PX (0.05% TDL and 0.5% TDL, respectively); Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate.

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et al., 1976) containing 0.5% mannitol as the carbon source. Cells were harvested at OD_{550} = 1.2. Inside-out vesicles containing EII^{mtl} were prepared as described by Reenstra et al. (1980). Membranes for EII^{mtl} purification were prepared as described by Jacobson et al. (1979). Both membrane preparations were kept in liquid N_2 until used.

Purification of EII^{mtl} was performed according to a modified procedure of Jacobson et al. (1979). Membranes from 14 g of cells were extracted at 25 °C by stirring in 200 mL of extraction buffer (0.5% sodium deoxycholate, 20 mM Tris-HCl, pH 8.4, 0.2 M NaCl, and 1 mM DTT). After 30 min the solution was chilled on ice, and all further operations were performed at 4 °C. The extracted membrane suspension was centrifuged (140000*g*, 65 min), and the supernatant was loaded on a column of hexylagarose (2 × 29 cm), preequilibrated in extraction buffer. The column was washed with 350 mL of extraction buffer. The EII activity was eluted with extraction buffer containing 0.5% Lubrol PX. The active fractions were pooled (46 mL) and diluted with 460 mL of extraction buffer. This solution was loaded on a second smaller hexylagarose column (2 × 11 cm). The column was washed with 150 mL of extraction buffer followed by a 150-mL 0.0–1.5% Lubrol PX gradient in extraction buffer. The peak of EII activity eluted at 0.6% Lubrol PX. The peak fractions were pooled (18 mL) and dialyzed for 30 h against 2 volumes of 1 L of 20 mM Tris-HCl, pH 8.4, containing 0.5% Lubrol PX and 1 mM DTT. The dialyzed material was diluted with 150 mL of 20 mM Tris-HCl, pH 8.4, and 1 mM DTT and loaded on a butylagarose column (2 × 9 cm), preequilibrated with 20 mM Tris-HCl, pH 8.4, 0.05% Lubrol PX, and 1 mM DTT (0.05% TDL). The column was washed with 60 mL of 0.05% TDL, followed by a 100-mL 0–100 mM NaCl gradient in 0.05% TDL. The peak of the active fractions eluted at 20 mM NaCl. The pool (16 mL) was incubated for 10 min at 30 °C in the presence of 5 mM DTT (see Results). The DTT concentration was lowered to 0.05 mM by subsequent dilution and concentration in an Amicon diaflow apparatus equipped with a YM-30 filter. This isolation procedure resulted in 2.5 mL of 3.5 μM EII. SDS (10%)–polyacrylamide gel electrophoresis showed a single band with an apparent molecular weight of 58 000. Lubrol PX (0.025–0.05%) was present in all experiments performed with EII^{mtl}.

Assays. EII activities were determined by an assay in which the rate of mannitol phosphorylation was linear with the EII concentration. EII was incubated at 30 °C in a buffer containing 25 mM Tris-HCl, pH 7.6, 10 mM NaF, 5 mM $MgCl_2$, 1 mM DTT, 0.05% Lubrol PX, 10 mM PEP, 0.1–1.0 mM [^{14}C]mannitol (4–0.4 mCi/mmol, respectively), and saturating amounts of EI and HPr. Aliquots were withdrawn at various time intervals, and the phosphorylated sugar was separated from nonphosphorylated sugar by the Dowex AG 1-X2 ion-exchange column procedure (Misset et al., 1980) with 0.1 N HCl in place of LiCl as the eluent.

EII concentrations were determined in a procedure which measures the amount of phosphorylated EII from [^{14}C]PEP (Misset et al., 1983). Various amounts of EII were incubated at 30 °C with 0.23 μM EI, 0.24 μM HPr, 50 mM sodium phosphate buffer, pH 7.0, 1 mM DTT, 0.5 mM $MgCl_2$, and 5 μM [1-^{14}C]PEP (12 mCi/mmol). The [^{14}C]pyruvate formed was separated from [^{14}C]PEP on an ion-exchange column (Brouwer et al., 1982). The pyruvate concentrations were corrected for the amounts arising from the phosphorylation and hydrolysis of phosphorylated EI and HPr, determined in the absence of EII. EII concentrations given in the text are always concentrations of available phosphoryl binding sites.

Table I: Influence of Reduced PMS, Fe^{3+}, and Phenylarsine Oxide on the EII^{mtl} Activity in Inverted Membrane Vesicles and on the Inhibition by NEM

	inverted vesicles incubated in the presence of	EII^{mtl} activity (%)	
		−NEM[a]	+NEM[b]
A	no additions	100	18
B	reduced PMS	4	80
C	KCN/reduced PMS	89	
D	Fe^{3+}	15	85
E	phenylarsine oxide	18	90

[a] EII^{mtl} activity was measured as described under Experimental Procedures except that DTT was omitted from the assay mixture. The final protein concentration of the inverted vesicles in the assay mixture was 0.5–3 μg/mL. All experiments were performed at 30 °C. (A) Reference, no additions. (B) Vesicles were incubated for 15 min in the presence of 10 mM ascorbate and 10 μM PMS under a continuous flow of oxygen. (C) As in (B), except that prior to the addition of reduced PMS the vesicles were incubated for 5 min with 1 mM KCN. (D, E) Vesicles were preincubated for 10 min with 6.3 mM $K_3Fe(CN)_6$ (D) or 2 mM phenylarsine oxide (E). EII^{mtl} activities were measured in the presence of 0.6 mM $K_3Fe(CN)_6$ and 0.2 mM phenylarsine oxide, respectively. [b] NEM treatment: Vesicles were incubated for 5 min with 1 mM NEM in the presence of (A) no additions, (B) 10 mM ascorbate and 10 μM PMS under a continuous flow of oxygen, (D) 6.3 mM $K_3Fe(CN)_6$, and (E) 2 mM phenylarsine oxide. After 5 min 25 mM DTT was added, and EII^{mtl} activities were measured.

Reaction of EII^{mtl} with [^{14}C]NEM. Stock [^{14}C]NEM was diluted 10-fold in pentane. Suitable aliquots were stored under nitrogen atmosphere in closed hydrolysis tubes. Prior to reaction with EII^{mtl} a given amount of [^{14}C]NEM in pentane was added to water. The pentane was removed from the water surface with a stream of dry N_2 gas. After reaction of EII^{mtl} with [^{14}C]NEM (see table footnotes for specific details), nonreacted [^{14}C]NEM was removed by extensive dialysis. The EII^{mtl}-containing samples were diluted in 0.5% TDL buffer to a final volume of 2.5 mL and dialyzed against three 500-mL volumes of 0.5% TDL buffer. Sephadex G-15 gel filtration showed that no free label remained in the dialyzed samples. Control experiments showed that no EII^{mtl} was absorbed on the dialysis membrane.

Radioactivity was counted in the presence of 8 mL of Packard emulsifier scintillator in a Nuclear Chicago Mark I liquid scintillation counter. The counting efficiency, determined by an internal standard, was 75%.

Results

Sulfhydryl Group Reactivity of Membrane-Bound EII^{mtl}. Table I shows that reduced PMS, Fe^{3+}, and phenylarsine oxide strongly inhibit EII^{mtl} activity in inverted cytoplasmic membrane vesicles from *E. coli* (Table I). The results parallel those reported earlier (Robillard & Konings, 1981; Robillard et al., 1983) for the activity of *E. coli* $EIIB^{Glc}$ in inverted vesicles. Preincubation of EII^{mtl}-containing membranes with KCN, which itself does not influence EII^{mtl} activity, prevents the inhibition of EII^{mtl} activity by reduced PMS. The inhibition of EII^{mtl} activity by reduced PMS, Fe^{3+}, and phenylarsine oxide was reversed by the addition of excess DTT (not shown). Incubation of EII^{mtl} vesicles with the sulfhydryl alkylating reagents NEM or bromopyruvate resulted in an inactive EII^{mtl} preparation. This inhibition was not reversed by DTT. When, prior to addition of NEM or bromopyruvate, the inside-out vesicles were preincubated with Fe^{3+}, there was no irreversible loss of EII^{mtl} activity. Similarly, preincubation with reduced PMS protected EII^{mtl} against NEM inhibition. From the similarities between the results obtained with EII^{mtl} and EII^{Glc} containing inside-out vesicles, we conclude, analogous to the

Table II: Label Incorporation in Urea-Denatured EII[a]

	sequence of additions to denatured EII	[^{14}C]NEM/EII (mol/mol)
A	[^{14}C]NEM/DTT	1.9
B	[^{14}C]NEM/DTT/AsO_2^-/[^{14}C]NEM/DTT	2.9
C	DTT/AsO_2^-/[^{14}C]NEM/DTT	2.9

[a] EII was denatured by the addition of urea followed by a 60-min incubation at 30 °C. Final concentrations: EII, 1.9 μM; urea, 7.8 M; sodium phosphate buffer, 35 mM, pH 7.0. [^{14}C]-NEM labeling of urea-denatured EII was performed at 30 °C in three different ways: (A) Incubation with 0.31 mM [^{14}C]NEM. After 10 min the labeling was stopped by addition of 18 mM DTT. (B) Incubation with subsequent addition of the following: t = 0 min, 0.31 mM [^{14}C]NEM; t = 10 min, 2.6 mM DTT; t = 30 min, 5 mM $NaAsO_2$; t = 32 min, 0.32 mM [^{14}C]NEM; t = 47 min, reaction stopped by addition of 13 mM DTT. (C) Identical with (B), except that the first addition of [^{14}C]NEM was replaced by H_2O. Label incorporation in EII determined by the dialysis method described under Experimental Procedures.

conclusion of Robillard & Konings (1980) for EII^{Glc}, that dithiol–disulfide interchanges play an important role in the functioning of EII^{mtl} in inside-out vesicles. The following experiments with purified EII^{mtl} were intended to answer two questions: (1) whether the redox interchanges involve some regulatory protein distinct from EII^{mtl} or EII^{mtl} itself, and (2) whether the interchanges regulate the activity of the enzyme or occur during the turnover of the enzyme.

Sulfhydryl–Disulfide Distribution in Denatured EII^{mtl}. The number of reactive thiol groups in EII^{mtl} was determined by denaturing the enzyme in 7.8 M urea, followed by reaction with [^{14}C]NEM. Table IIA shows that denatured EII^{mtl} contains two reactive sulfhydryls. The method of Zahler & Cleland (1968) was used to determine whether EII^{mtl} contained any disulfide bridges. According to this method the available disulfide bridges in the urea-treated protein are first reduced by an excess of DTT. The excess DTT is removed by reaction with arsenite, which complexes dithiols forming cyclic dithiol arsenites. [^{14}C]NEM is then reacted with the thiol groups originating from the disulfides. When this method was applied to denatured EII^{mtl} in which the two sulfhydryls were already blocked with [^{14}C]NEM, we found that only one extra thiol could be labeled (Table IIB). Since this extra thiol must originate from a disulfide bridge, the results suggest that the denatured EII^{mtl} preparation consists of two EII^{mtl} monomers connected by an intermolecular disulfide bridge, where the monomer is defined as that unit which can be phosphorylated. Direct reduction of denatured EII^{mtl} before NEM treatment leads to the same label incorporation as in the above experiment (Table IIC).

Sulfhydryl Group Reactivity and Sulfhydryl–Disulfide Distribution in Native EII^{mtl}. The EII^{mtl} purification procedure results in a concentrated EII preparation (2.5–4 μM) that is very sensitive to inactivation by NEM. The enzyme is inactivated after a 2-min incubation with 0.3 mM NEM. It is important to note that if the incubation with 5 mM DTT after the butylagarose column elution was omitted in the isolation procedure, the concentrated EII preparation could only be inactivated by high NEM concentrations (>1 mM) in the presence of substantial amounts of DTT (>0.4 mM). Further, if DTT was omitted from the assay mixture, the apparent activity of such a preparation was less than 40% of the activity in the presence of 1 mM DTT. These results indicate that the enzymatic properties of EII^{mtl} strongly depend on its redox state. For this reason all experiments described in this report are performed with EII^{mtl} preparations purified as described under Experimental Procedures, including the DTT preincubation before concentration.

Table III: Incorporation of [^{14}C]NEM in Native EII^{mtl} and P-EII^{mtl} [a]

	sequence of additions	EII activity[b] (%)	[^{14}C]-NEM/EII (mol/mol)
A	EII + [^{14}C]NEM	8	1.2
B	EII + phenylarsine oxide + [^{14}C]NEM	80	0.3
C	P-EII + [^{14}C]NEM	97	1.0
D	P-EII + [^{14}C]NEM + mannitol	8	1.8
E	P-EII + phenylarsine oxide + [^{14}C]NEM	90	0.2
F	E-II + [^{14}C]NEM + P-HPr	10	1.2

[a] (A, B) Labeling of EII^{mtl}. (A) EII^{mtl} was reacted with [^{14}C]NEM at 30 °C in a mixture containing 2.3 μM EII^{mtl}, 33 mM sodium phosphate buffer, pH 7.0, 0.6 mM $MgCl_2$, and 0.44 mM [^{14}C]NEM. After 2 min the labeling was stopped by diluting 15 μL of the reaction mixture in 1.0 mL of 0.5% TDL. (B) Same conditions as in (A), except that EII^{mtl} was preincubated for 2 min with 2.5 mM phenylarsine oxide before [^{14}C]NEM was added. The labeling was stopped by addition of 14 mM DTT after 10 min. (C–F) Labeling of P-EII^{mtl}. EII was phosphorylated in a reaction mixture containing 33 mM sodium phosphate buffer, pH 7.0, 0.6 mM $MgCl_2$, 3.3 mM PEP, 7.9 nM EI, and 4 μM HPr. Temperature was 30 °C. In all experiments the final EII concentration was 1.4 μM. Labeling reactions were stopped by the addition of 14 mM DTT. (C) P-EII incubated with 0.31 mM [^{14}C]NEM for 6 min. (D) At zero time [^{14}C]NEM (0.31 mM) was added to P-EII. At t = 6 min mannitol was added to a final concentration of 0.7 mM. At t = 14 min DTT was added. (E) P-EII for 2 min incubated with 2.5 mM phenylarsine oxide. At t = 2 min 0.34 mM [^{14}C]NEM was added. DTT was added at t = 8 min. (F) At zero time [^{14}C]NEM (0.38 mM) was added to 1.8 μM EII in 33 mM sodium phosphate buffer, pH 7.0, containing 0.6 mM $MgCl_2$. At t = 3 min the other PTS components PEP, HPr, and EI, were added. Final concentrations of all components were the same as given above. Final [^{14}C]NEM concentration 0.31 mM. Enzyme II activities and EII bound radioactivity were determined as described under Experimental Procedures.
[b] EII activity was expressed as percentage of an untreated control sample.

When native EII^{mtl} is incubated for 2.5 min with 0.44 mM [^{14}C]NEM, complete inactivation occurs, and 1.2 [^{14}C]NEM labels per EII are incorporated (Table IIIA). Prolonging the incubation for 15 min did not increase the label incorporation. These data suggest that only one of the two thiols which occur in denatured EII^{mtl} is readily alkylated by [^{14}C]NEM in native EII. The rate of reaction of the second thiol is by comparison much slower.

The data presented at the beginning of the Results show that the oxidizing reagent K_3FeCN_6 and the dithiol blocking reagent phenylarsine oxide inhibit the activity of EII^{mtl}-containing vesicles and protect the enzyme against NEM inactivation. The same kind of inhibition and protection was studied with purified EII^{mtl} (Table IV). Fe^{3+} and phenylarsine oxide strongly inhibit the activity of purified EII^{mtl}; if the addition of DTT in the experiments in Table IVE,G was omitted, the EII activity after preincubation was less than 10% of the control value. The inhibition of EII^{mtl} activity by Fe^{3+} is not completely reversed by DTT. This is probably due to a nonspecific salt effect because the same partial nonreversibility occurs after incubation with Fe^{2+}. Preincubation of EII^{mtl} with Fe^{3+} protects the enzyme against NEM activation for 85% (compare Table IVE,F). In contrast preincubation of EII with Fe^{2+} did not result in any protection against NEM

Table IV: Inactivation of EII^{mtl} by NEM in the Presence of Fe^{3+}, Fe^{2+}, and Phenylarsine Oxide[a]

	EII preincubated with	NEM added	EII activity after DTT addition (%)
A		−	100
B		+	10
C	Fe^{2+}	−	73
D	Fe^{2+}	+	8
E	Fe^{3+}	−	73
F	Fe^{3+}	+	62
G	phenylarsine oxide	−	90
H	phenylarsine oxide	+	83

[a] EII^{mtl} at 0.25 μM in 33 mM sodium phosphate buffer, containing 0.5 mM $MgCl_2$, was incubated at 30 °C for 5 min with 1.6 mM K_4FeCN_6 (C, D) or 1.6 mM K_3FeCN_6 (E, F) and for 2 min with 2.5 mM phenylarsine oxide (G, H). After this preincubation 0.3 mM NEM was added to the indicated samples. DTT at 14 mM was added to samples A–F after 2.5 min and to samples G and H after 5 min. EII activities were assayed as described under Experimental Procedures and expressed as percentage of the control sample (A).

activation. The inhibition of EII^{mtl} by phenylarsine oxide was almost completely reversed by addition of DTT. Preincubation of EII with phenylarsine oxide protected the enzyme for more than 90% against NEM inactivation. When EII was preincubated for 2 min with 2.5 mM phenylarsine oxide followed by a 10-min incubation with 0.4 mM [^{14}C]NEM, 80% of the enzymatic activity was retained, and only 0.3 mol of label was incorporated per EII (Table IIIB). The protection afforded by Fe^{3+} and phenylarsine oxide indicates the presence of two adjacent thiols which can be converted to as a disulfide and thereby be protected from reaction with NEM.

Sulfhydryl Group Reactivity and Sulfhydryl–Disulfide Distribution in P–EII. Addition of EII to a mixture of [^{14}C]PEP, EI, and HPr results in a burst of [^{14}C]pyruvate due to the formation of P–EII (see Figure 1). Kinetic studies have shown that this intermediate is a catalytically significant species in the reaction leading to mannitol-P (F. F. Roossien et al., unpublished results). Under conditions where the EII-catalyzed mannitol phosphorylation is inhibitied by Fe^{3+} or NEM, 70% of the EII phosphorylation sites can still be phosphorylated (Figure 1). Apparently the dephosphorylation reaction or the binding of the sugar is inhibited by oxidants or NEM. Phosphorylation of EII^{Glc} protects the enzyme against NEM activation (Haguenauer-Tapis & Kepes, 1977; Robillard & Konings, 1981). Table IIIC shows that the same holds for purified EII^{mtl}; virtually no activity loss is observed when phosphorylated EII is incubated with 0.3 mM NEM. Even raising the NEM concentration to 1 mM did not result in considerable loss of EII activity (not shown). Labeling experiments with [^{14}C]NEM demonstrate that despite the retention of EII activity, one label is still incorporated in P–EII. Incorporation of [^{14}C]NEM in P–EII can be prevented by preincubation with phenylarsine oxide (cf. Table IIIE) or with Fe^{3+} (data not shown). This protection again suggests that a second cysteine must be located in the vicinity of the reactive sulfhydryl such that formation of a disulfide or cyclic dithiol arsenite is possible. Reaction of P–EII with NEM does not inactivate while reaction with dephosphorylated EII does inactivate. We would expect, therefore, that mannitol-induced dephosphorylation of singly labeled P–EII in the presence of [^{14}C]NEM should result in incorporation of a second label and inactivation. The data in Table IIID show that inactivation is accompanied by an additional incorporation of 0.8 mol of label per EII. The analogous experiment, the phosphorylation

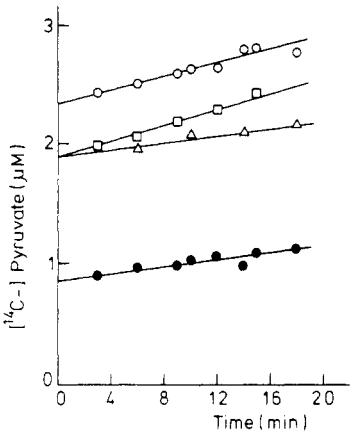

FIGURE 1: Pyruvate burst experiments performed as described under Experimental Procedures except that the [^{14}C]PEP concentration was 4.5 μM and EII was subjected to different preincubations. (●) No EII added, the formation of [^{14}C]Pyr originates from the phosphorylation of EI and HPr. (○) Control, EII not subjected to preincubation. (△) EII preincubated for 2 min with 0.33 mM NEM. The inactivation was stopped by addition of 14 mM DTT. (□) EII preincubated for 8 min with 5 mM K_3FeCN_6. The final Fe^{3+} concentration in the pyruvate burst assay is 1 mM. The formed [^{14}C]pyruvate is expressed as percentage of the original [^{14}C]PEP concentration.

of singly labeled inactive EII, followed by an additional incubation of [^{14}C]NEM did not lead to the incorporation of a second mole of label. Therefore, the cysteine which becomes accessible upon phosphorylation of active EII does not react upon phosphorylation of the NEM-inactivated singly labeled enzyme.

Discussion

EII^{mtl} activity in inverted cytoplasmic membrane vesicles can be protected from inhibition from sulfhydryl reagents by prior exposure to oxidizable substrates, oxidizing agents, or the dithiol-specific reagent phenylarsine oxide. Since oxidizing agents and phenylarsine oxide exert the same protective effect on purified EII^{mtl} as on the membrane-associated enzyme, we conclude that these agents and oxidizable substrates work directly at the level of EII^{mtl} in the membrane instead of at the level of a regulatory protein which controls EII^{mtl} activity.

The stoichiometry of label incorporation is based on an active site titration in which EII is phosphorylated from [^{14}C]PEP, producing a stoichiometric quantity of [^{14}C]pyruvate. In a separate study we have shown that essentially all of the phosphoryl groups on $P–EII^{mtl}$ can be converted to mannitol-P (F. F. Roossien et al., unpublished results). An EII^{mtl} monomer is then defined as that unit which accepts one phosphoryl group. A total of three sites can be labeled per EII^{mtl} monomer in the reduced denatured state. Since only two can be labeled without reduction, one is forced to suggest that the third site is a disulfide bridge between two monomers in the native enzyme. Each monomer in the native enzyme also contains two sulfhydryls, SH(A) and SH(B), capable of being alkylated. SH(A) is essential for enzymatic activity and is readily accessible only in the nonphosphorylated enzyme (see Figure 2). We have never observed alkylation of SH(A) in $P–EII^{mtl}$. SH(B) is most readily accessible in the phosphorylated enzyme. However, some label incorporation is observed at high NEM concentrtions or long exposure times even in the nonphosphorylated state (Table III). The experiments with oxidants and phenylarsine oxide suggest that the SH groups occur in pairs capable of being oxidized to disulfides. The simplest construction consistent with the observed patterns of protection would be disulfides between the subunits involving

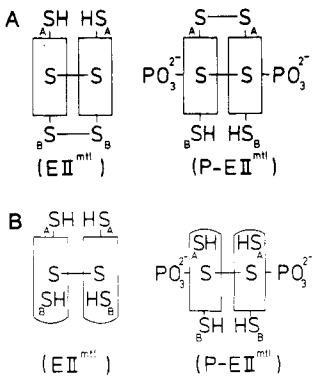

FIGURE 2: (A) Redox model consistent with the observed reactivities of enzyme II^{mtl} sulfhydryl groups toward [^{14}C]NEM. (b) Buried–exposed model consistent with the observed reactivities of enzyme II^{mtl} sulfhydryl groups toward [^{14}C]NEM.

the same sites [S(A)–S(A) or S(B)–S(B)]. This would be consistent with the apparent symmetry of the system (i.e., a dimer containing two active sites). Other constructions, however, involving intrasubunit disulfides (S_A–S_B) cannot be excluded.

SH(A) is protected in P-EII^{mtl}, and SH(B) is protected in the nonphosphorylated enzyme. The pattern of protection observed upon changing the phosphorylation state could be explained by an oxidation–reduction model or by a buried–exposed model. In a redox model (Figure 2A) phosphorylation of the enzyme alters the redox state of the vicinal thiols. For instance thiols A are reduced while thiols B are oxidized in EII. Upon phosphorylation thiols B become reduced and thiols A will be oxidized. Phosphorylation alone is capable of causing such a shift in redox states. Binding of a negatively charged phosphoryl group close to vicinal thiols would alter the apparent pK of these groups and, consequently, the midpoint potential of a dithiol–disulfide transition. At a constant redox potential phosphorylation of EII^{mtl} at site B could shift this site from the oxidized to the reduced form. If electron exchange occurs between the two sites, site A could provide the electrons for the reduction of site B. The net effect of phosphorylation near site B would be the oxidation of A to a disulfide and the reduction of B to a dithiol. Dephosphorylation would reestablish the original redox equilibrium (Robillard & Konings, 1982). In a buried–exposed model (Figure 2B) phosphorylation alters the accessibility, but not the redox state, such that SH(A) is exposed in EII but buried in P-EII while the opposite holds for SH(B) (see Figure 2). At the present time there is no conclusive evidence that phosphorylation alters the redox state of the dithiols. Neverthless the effects of phosphorylation/dephosphorylation are so similar to those of oxidation/reduction that such a mechanism must be seriously considered.

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